Chemical and biological sensors

Bacteria-Based Biosensor for the Detection of *Lactococcus Lactis* Bacteriophage in Agrifood Industry

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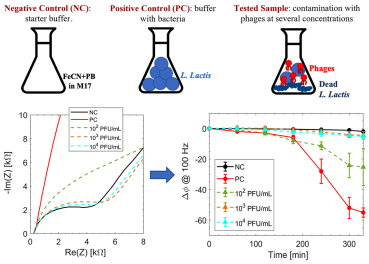
Abstract—This letter explores and clarifies the sensing mechanism of a proposed biosensor designed for the rapid detection of the phages of *Lactococcus lactis* in milk-based solutions. A dedicated experimental test revealed that the increased charge transfer resistance visible when *L. lactis* proliferates in solution results from both bacterial electrode coverage and direct bacterial interaction with redox species, most likely related to $Fe(CN_6)^{3-}$ consumption during *L. lactis* metabolism. Finally, the evaluation of biosensor performance through impedance phase shift analysis at 100 Hz offers a simplified approach for practical applications. The biosensor demonstrates efficient phage detection within 4 h with detection capabilities for phage concentrations $>10^3$ PFU/mL, and future work aims to validate its on-field applicability in authentic milk samples.



Index Terms—Chemical and biological sensors, electrochemical biosensor, electrochemical impedance spectroscopy (EIS), impedance phase, *Lactococcus lactis*, phage detection, sensing mechanism.

I. INTRODUCTION

Lactococcus lactis is a prevalent lactic acid bacterium, which is crucial for milk fermentation in the dairy industry due to its metabolic activity [1]. Nowadays, the agri-food sector is facing significant challenges on the timely identification of *L. lactis* bacteriophages, which is pivotal in preventing disruptions in the dairy production chain and subsequent financial and resource wastage [2]. Currently, microbiological laboratory techniques are mostly employed for detecting the phage spread in the production plants with the limitation of being costly, time-consuming with detection times exceeding 6 h, and requiring specialized equipment and personnel.

Recent works report various alternative biosensing solutions tailored for the dairy industry [3], [4], [5], [6], [7], [8], [9], [10], [11], addressing concerns such as the detection of health-hazardous bacteria in milk samples [8]. Typically, bacteriophages serve as recognition elements in biosensors designed for the detection of their corresponding bacteria [12]. However, only a limited number of studies have explored biosensing solutions for directly detecting the phages themselves [13]. In our previous work [11], the method for detecting *L. lactis* phages was validated using milk-based samples, able to detect relatively low concentrations of phages, specifically 10^3 plaque forming units/mL (PFU/mL) in laboratory-prepared solutions. The sensing method used electrochemical electrodes on which was grown living *L. lactis* bacteria. Electrical proprieties, as the electrochemical impedance response, change with the growth or lysis of the *L. lactis* bacteria, which were used to detect the presence of their related phages.

Within this letter, we further clarify the sensing mechanism behind the *L. lactis* phage biosensor proposed in previous work [11]. Further experimental results indicated that the increase in the charge transfer resistance may be induced not only by the increased bacterial coverage of the electrodes but also by a direct interaction of the bacteria with the redox species, most likely related to the consumption of $Fe(CN_6)^{3-}$ in the metabolism of *L. lactis*. Finally, the biosensor performance is evaluated by the analysis of the impedance phase shift at a single frequency, 100 Hz, allowing the simplification of the read-out circuits and signal conditioning setup for its use in the final applications.

II. MATERIALS AND METHODS

A. Pathogens, Chemicals, and Biosensor Electrodes

The *L. lactis*, provided by DSMZ, Germany, was cultured using M17 broth, while its bacteriophage P008 was grown in a saline phage buffer (pH 7.5) composed of 100 mM NaCl, 8 mM $MgSO_4$, and 50 mM Tris-HCl. Milk was commercial off-the-shelf milk. $CaCl_2$ was also added to the testing solution at 10 mM to enhance the phage lysis, thus improving the final sensing performances [14], [15], [16], [17]. Potassium hexacyanoferrate III ($K_3[Fe(CN)_6]$), potassium hexacyanoferrate II ($K_4[Fe(CN)_6]$) trihydrate and calcium chloride ($CaCl_2$) were diluted at 5 mM in the M17 for the electrochemical measurements. Solutions containing the 5 mM redox $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ will be called FeCN solution for brevity. More details on the preparation procedure of the solutions can be found in [11].

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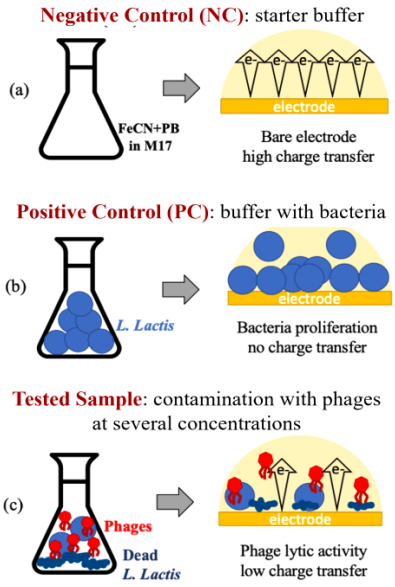

Fig. 1. Schematic representation of the main types of experiments that are carried out. (a) NC with a solution containing only the filtered milk, M17, phage buffer, and the FeCN. (b) PC with the NC solution and living *L. lactis*. (c) Tested sample with the PC solution and the addition of several concentrations of phages.

The proposed biosensors were based on screen-printed electrodes, model DRPC223AT, Metrohm DropSens, consisting of working (Au), counter (Au), and reference (Ag) electrodes on a ceramic substrate measuring 34 mm × 10 mm × 0.5 mm. Electrochemical impedance spectroscopy (EIS) measurements were conducted by placing the devices in a custom 3D-printed polydimethylsiloxane cell to prevent unintended evaporation [10]. EIS was performed in a frequency range from 1 Hz to 100 kHz, with a V_{ac} signal peak-to-peak amplitude of 10 mV and a V_{dc} bias of 91 mV, which corresponds to the equilibrium potential.

B. Experimental Setup

The experimental setup is represented in Fig. 1, which shows the three main types of experiments carried out in parallel. The negative control (NC) consisted of measuring the biosensor response in time with a solution containing only the filtered milk, M17, phage buffer and FeCN. The measurements showed modest resistance to the charge transfer, due to the presence of the redox FeCN species. The positive control (PC) consisted of measuring the biosensor response in time with the NC solution and living *L. lactis* bacteria. Depending on the level of growth of the bacteria, the biosensor response was characterized by high resistance to the charge transfer. Finally, the tested sample consisted of the PC solution with the addition of several concentrations of phages. All concentrations are expressed in PFU/mL. Depending on the concentration of phages, the *L. lactis* growth was slowed down or inhibited by the phages, which are detected as changes in the charge transfer resistance [10].

III. RESULTS AND DISCUSSION

A. EIS Response

Fig. 2 compares sensors tested with the phage-contaminated solution (blue lines) and with the *L. lactis* control solution (red lines) to assess the bacterial growth. The Nyquist curves and the impedance magnitude and phase clearly show the different responses of the sensors. At time 0 min, all curves exhibit similar shapes. After 330 min, the curves of the sensors having the phage contamination show only slight increase, similar to NC, whose slight increase is due to the sensor stability [13], as well as contributions from undesired deposits on the sensor surface. In contrast, the PC sensor impedance increases enormously, due to large increases of the charge transfer resistance (R_{ct}). These differences in electrochemical behavior prove the biosensor capability to detect the presence of *L. lactis* phages in milk-based solutions.

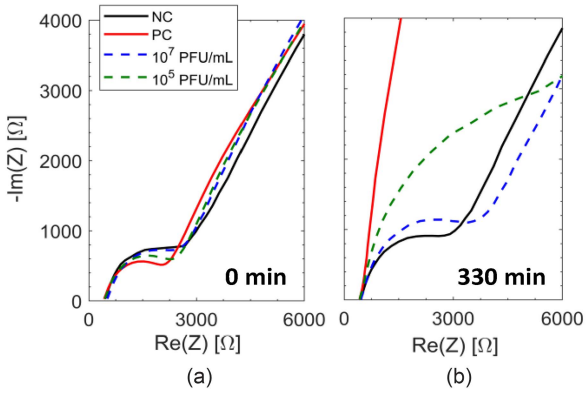

Fig. 2. Nyquist plots of the EIS response of several sensors. NC, PC, and with test samples contaminated with 10^7 and 10^5 PFU/ml of phages (no $CaCl_2$). Measurements are performed (a) at 0 min (immediately after the preparation of the solutions) and (b) after 330 min.

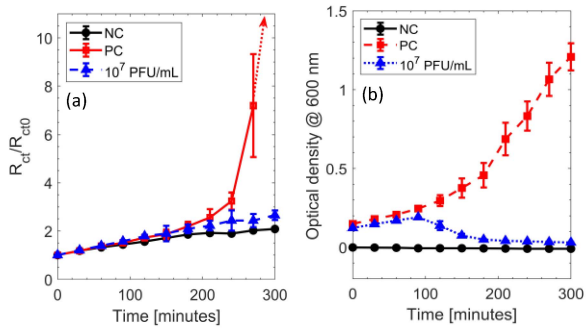

Fig. 3. (a) Normalized R_{ct} and (b) optical density (absorbance) at 600 nm as a function of the time of NC, PC, and 10^7 PFU/mL samples.

B. Investigation of the Cause of R_{ct} Increase

As noted in [11], the increase of the R_{ct} was mainly attributed to the coverage of the *L. lactis* on the surface of the electrodes. However, the kinetics of the R_{ct} curves for solutions contaminated with the phages clearly evidence a monotonic trend also for the solutions contaminated with high concentrations of phages [11], i.e., 10^7 PFU/mL shown in Fig. 3(a). At high time points, the continuous slight increase in 10^7 PFU/mL could be primarily attributed to the same phenomenon causing the increase in R_{ct} over time in NC, since the slopes of these two curves are identical. This monotonic trend of R_{ct} differs from optical absorbance measurements at 600 nm, which were conducted to monitor the growth of the *L. lactis* bacteria in the solutions. Indeed, as shown in Fig. 3, optical measurements evidence a clear decrease in the absorbance at times > 200 min, due to the lytic action of phages, which decreases the living bacteria population and dominates over the bacterial growth. So, we investigated the causes of this continuous increase in the R_{ct} of sensors.

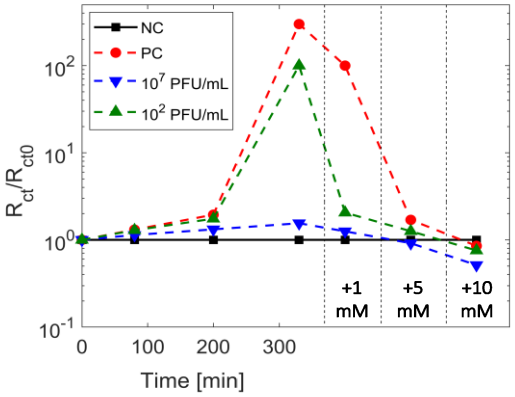

Fig. 4. Normalized charge transfer resistance R_{ct}/R_{ct0} extrapolated by the EIS measurements as a function of the time. At 0 min, the solution contains 5 mM FeCN. After 330 min, the biosensor response was measured by adding +1, +5, and +10 mM of $Fe(CN_6)^{3-}$/ $Fe(CN_6)^{4-}$ to the initial solutions.

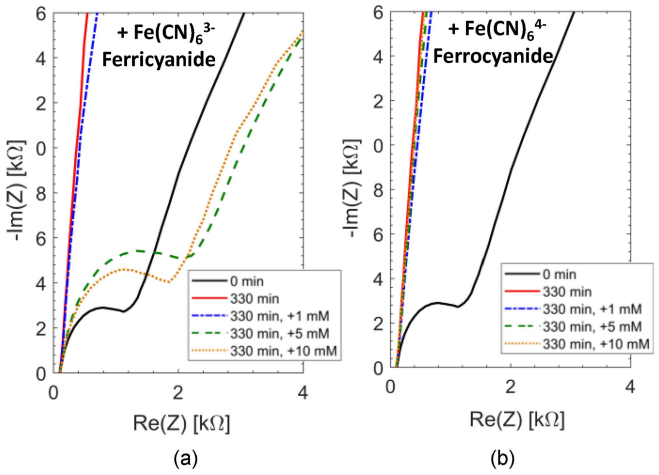

Fig. 5. EIS of the positive control (PC) with 5 mM FeCN at time 0 min. After 330 min, the response was measured by adding +1, +5, and +10 mM of (a) only ferricyanide $Fe(CN_6)^{3-}$ and (b) only ferrocyanide $Fe(CN_6)^{4-}$ to the initial solutions. (c) Schematic representation of the metabolic reactions of *L. Lactis* inducing variations of the concentration of the ferricyanide $Fe(CN_6)^{3-}$ and ferrocyanide $Fe(CN_6)^{4-}$.

Fig. 4 reports the normalized $R_{ct}/R_{ct\,0}$ by the EIS measurements as a function of the time. The R_{ct} values were extrapolated by using an equivalent electric circuit of the Randles cell with a constant phase element. As expected, the PC and the low-concentrated phage solution exhibit a large increase of the charge transfer resistance, which increases by almost × 100. After the test, the biosensor responses were measured by adding +1, +5, and +10 mM of $Fe(CN_6)^{3-}$ /$Fe(CN_6)^{4-}$ to the initial solutions. The PC and low-concentrated phage sensors recovered their R_{ct}, as it started to decrease, thus indicating that the R_{ct} was mostly limited to the absence of FeCN. Moreover, tests were also carried out with sensors in a vertical position to avoid any type of deposition on the electrode surface. Vertically positioned sensors still demonstrated a large increase in the R_{ct} for PC, having about 70× increase versus >100 × increase of horizontal sensors.

To understand the nature of the increased R_{ct} phenomenon, we performed EIS measurements on PC sensors comparing the initial responses (0 min) with the responses at 330 min without the addition and after the addition of only ferricyanide, reported in Fig. 5(a), and only

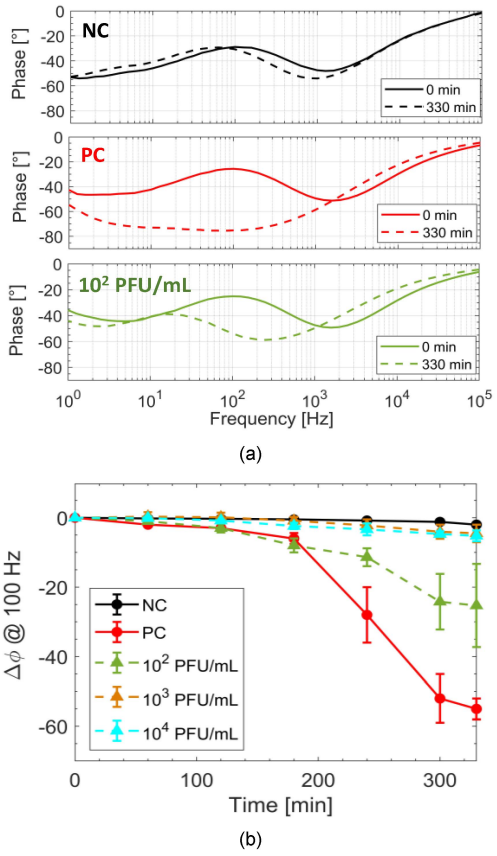

Fig. 6. (a) Phases of EIS responses as a function of frequency of NC, PC, and 10^2 PFU/mL in filtered milk-based solutions with $CaCl_2$ at 0 and 330 min. (b) Phase shift measured at 100 Hz as a function of the time for different samples and different phage concentrations.

ferrocyanide, reported in Fig. 5(b). Notably, with +5 mM ferricyanide, the EIS signal recovers quickly, restoring the semicircle shape with an R_{ct} ~6.8 kΩ. An even more evident recovery is obtained for +10 mM ferricyanide, where R_{ct} is ~5.7 kΩ. Meanwhile, little to no variation of the EIS signal is visible with any addition of ferrocyanide after 330 min. These results suggest an absence of the $Fe(CN)_6^{3-}$ in PC, most likely due to the oxidized specie involvement in the metabolism of the *L. lactis* bacteria, which can exploit some electrons acceptors, such as ferricyanide for their glucose metabolism [18]. Due to this metabolic consumption, the EIS signal is affected by the lack of oxidized specie and can be restored only after its addition in solution. Moreover, the sensor specificity should be enhanced by this phenomenon since this metabolic pathway is characteristics of these bacteria.

C. Sensing Performances Using the Phase Shift

The extrapolation of R_{ct} can be difficult at high bacterial growth, as for PC, also requiring a full impedance scan in the frequency span. Another discriminative parameter can be the impedance phase. Fig. 6 shows the impedance phase for tests with $CaCl_2$-treated milk-based samples. At time 0 min, all the curves show similar shapes. After 330 min, the PC curve shows the largest decrease in the phase in the medium frequencies, having the maximum phase difference Δf at 100 Hz, about −55° for PC, <−1° for NC, and −24° for 10^2 PFU/mL sample. This suggests that the sensing method can potentially be simplified by performing the impedance measurement at a single frequency: 100 Hz. Fig. 6(b) shows the phase shifts retrieved by the

impedance measurement at the single frequency of 100 Hz for NC, PC, and at several concentrations of phages. The curves at phage concentrations of 10^3 and 10^4 PFU/mL have distinguishable trends from the PC. Meanwhile, $\Delta\phi$ for 10^2 PFU/mL shows high variability, suggesting detection capabilities above 10^3 PFU/mL. It is worth noting that the lowest phage contamination actively affecting dairy production is reportedly around 10^5–10^4 PFU/mL [19], [20]. Hence, the detection limit of the proposed sensor is at least one order of magnitude lower than the final application minimum requirement.

IV. CONCLUSION

In this letter, we delve deeper into elucidating the sensing mechanism underlying the *L. lactis* phage biosensor previously proposed in the prior study. Additional experimental findings suggest that the increase of charge transfer resistance may arise not solely from the coverage of the bacteria on the sensor electrodes but also from a direct interaction between the bacteria and the redox species, most likely associated with the utilization of $Fe(CN_6)^{3-}$ in the metabolism of *L. lactis*.

Finally, the biosensor's performance is assessed through the examination of impedance phase shifts at a specific frequency, 100 Hz. This approach enables the simplification of read-out circuits and signal conditioning setups, facilitating its practical implementation in the final applications. Phage detection can be completed within a timeframe of under 4 h, with observable variations in the phase shift ranging from $-10°$ to $-55°$ when compared with the positive control. Subsequent experiments will focus on evaluating the sensor's efficacy in authentic milk samples under challenging conditions, with the ultimate goal of validating its suitability for on-field applications.

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